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# RAPID COMMUNICATION

# Patterns of *in Vitro* Anti-Human Immunodeficiency Virus Type 1 Antibody Production in Long-Term Nonprogressors[1]

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**With the aim of evaluating the specific pattern of *in vitro* antibody production (IVAP) in human immunodeficiency virus type 1 (HIV-1)-infected long-term nonprogressors (LTNPs), we tested 20 subjects who had remained asymptomatic for more than 8 years with a CD4$^+$ cell count higher than 500/$\mu$l and 59 patients at different stages of HIV-1 infection as controls. In cell cultures, IVAP was detected in 14 out of 20 LTNPs (70%), in 5 out of 6 recent seroconverters (83%), and in all the other control patients. Anti-p24 antibody production was significantly lower in LTNPs than in asymptomatic patients with a more recent infection. Recent seroconverters and patients with AIDS did not produce anti-p24 antibodies ($P = 0.02$). Anti-gp160 antibodies were produced by peripheral blood mononuclear cells from LTNPs in 12/20 cases. CD4$^+$ cell count was significantly higher in IVAP-negative than in IVAP-positive LTNPs ($P = 0.013$), while the viral load was not significantly different. Specific anti-HIV-1 antibody production did not seem to be a correlate of long-term nonprogression.** 

***Key Words:*** **HIV-1; IVAP; anti-p24.**

## INTRODUCTION

In the natural course of clinical HIV-1 infection, a minority of human immunodeficiency virus type 1 (HIV-1)-infected subjects remain asymptomatic for a long period of time with stable CD4 cell counts ≥500/$\mu$l (1–3). These subjects are currently defined as long-term nonprogressors (LTNPs). It is believed that virus control in LTNPs might be either the result of infection with defective viruses (4) or the presence of a strong cellular immune response (5, 6).

The role of the humoral response in nonprogression has been mainly investigated by analyzing the production of neutralizing antibodies, without conclusive results (7, 8). Another possible approach in evaluating the humoral response in LTNPs is represented by the study of anti-HIV-1 specific *in vitro* antibody production (IVAP) (9, 10). We recently described a different anti-HIV-1 antibody pattern in various phases of HIV-1 infection, suggesting a lack of anti-*core* IVAP in the progression of HIV-1 infection (11).

The aim of this study was, first, to investigate whether LTNPs show a specific pattern of IVAP and, second, to verify the potential role of an efficient anti-*core* IVAP as a protective factor.

## MATERIALS AND METHODS

### *Study Subjects*

Twenty subjects with long-term nonprogressive infection were studied. The criteria used to define nonprogression included documented HIV-1 infection for more than 8 years, stable CD4$^+$ cell counts greater than 500/$\mu$l, the absence of symptoms, and no antiretroviral treatment. The patients were consecutively enrolled at the Institute of Infectious Diseases, University of Milan, Luigi Sacco Hospital, Milan. CD4$^+$ cell counts were determined using a Coulter EPICS Elite ESP flow cytometer (Coulter Corp., Miami, FL). All but 2 patients were previous intravenous drug users between the ages of 28 and 41, and the remaining 2 patients had been infected through sexual contacts. Among the subjects, 14 were males and 6 females. As four control groups, we examined 6 recent seroconverters who had been infected within 6 months of enrollment, 15 asymptomatic patients with CD4$^+$ cell counts greater than 500/$\mu$l, 30 asymptomatic patients with CD4$^+$ cell counts between 200 and 500/$\mu$l (48% antiretroviral therapy

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experienced), and 8 patients with AIDS being treated with antiretroviral drugs with a CD4 count lower than 200/$\mu$l. Control patients were matched with LTNPs for gender, age, and risk factor. Several of them had been included in a previous study on *in vitro* antibody production (11). None of the patients had been treated with highly active antiretroviral therapy. We also examined 5 healthy subjects who were seronegative for anti-HIV-1 antibodies in order to rule out aspecific reactions. Serum from an HIV-1-positive patient with a complete Western blot (WB) pattern was used as a control for antibody reactivity.

### *Preparation of Peripheral Blood Mononuclear Cells and Cellular Assays*

Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll–Paque gradient centrifugation and resuspended at $1.5 \times 10^6$ /ml in RPMI medium, supplemented with 20% fetal calf serum, 1% L-glutamine, and 50 mg/ml gentamycin (R-20). PBMC cultures were incubated with phytohemagglutinin at 10 $\mu$g/ml for 8 days at 37°C in a moist 7% $CO_2$ atmosphere. PBMC were cultured in the presence or absence of 100 $\mu$g/ml pokeweed mitogen (PWM; Sigma BioSciences, St. Louis, MO). WB assay (Diagnostic Biotechnology Ltd., Singapore) was performed on PBMC culture supernatants. Serum levels of anti-p24 antibodies (Abs) and p24 antigen were determined. Isolation of HIV-1 from PBMC was performed through a standard cocultivation (12). HIV-1 isolates were checked for syncytium-inducing (SI) or non-syncytium-inducing (NSI) phenotype (13).

### *HIV-1 RNA Analysis*

The HIV-1 genomic RNA quantitation (copies/ml of plasma) was determined in LTNPs according to the method of Bagnarelli and colleagues (14). Briefly, the *gag* fragment (nucleotides 1551 to 1665) was analyzed with primer pair SK38 and SK39 in each sample. Competitive analysis was performed by using the plasmid pSKAN in each sample, as an internal competitor, in which the *gag* fragment was changed with an 18-bp downstream deletion from the T3 RNA polymerase promoter.

### *Statistical Analysis*

We analyzed our results with statistical tests using the Systat computer software program for Macintosh, version 5.1 (Evanston, IL). We first correlated IVAP, either anti-*core* or anti-*env* Abs production, along with the possibility of isolating HIV-1 from PBMC (Fisher's exact test). Secondly, we compared the plasma viremia and $CD4^+$ cell counts in IVAP-positive and -negative subjects (Mann–Whitney test). Data from the different control groups were also compared using a $\chi^2$ for linear trend test, Fisher's exact test, and Student's *t* test for paired data.

## RESULTS

Table 1 shows the pattern of *in vitro* anti-HIV-1 antibody production in the groups of patients we studied. PBMC from LTNPs produced anti-HIV-1 Abs without PWM stimulation less frequently than the control patients (14/20 vs 58/59, $P < 0.001$). Moreover, anti-*env* Abs production was significantly lower in LTNPs ($P < 0.001$). Anti-gp120 and gp-41 Abs were undetectable in culture supernatants of LTNPs. On the contrary, anti-gp120 and anti-gp41 were seen in 40 and 8% of the patients in asymptomatic subjects, respectively. The production of anti-p24 Abs was present in 60% of asymptomatic subjects with $\geqslant$500 $CD4^+/\mu$l, 30% of asymptomatic subjects with $\geqslant$200 to $<$500 $CD4^+/\mu$l, and 15% of LTNPs, and was absent in recent seroconverters and symptomatic subjects (Fisher's exact test; $P = 0.02$). PWM stimulation of cultured lymphocytes abolished antibody production in 18% of cultures in which a PWM-unstimulated IVAP was detected, and in another 41% we observed a loss of one or more specific antibodies (e.g., anti-gp120) without any significant difference among the different groups, as assessed by WB.

Twelve of the 20 LTNP patients showed a production of Abs against the envelope proteins gp160; 6 subjects were IVAP-negative, whereas 1 patient produced only anti-p55 Abs and another produced only anti-p24 Abs (Table 2). Only one LTNP was able to produce anti-p24, anti-p55, and anti-gp160 Abs. PWM stimulation did not induce any variation in Abs secretions. All of the patients were positive for anti-p24 Abs on serum and, with 1 exception, were negative for p24 antigen on serum. We obtained a viral isolation in 11 out of the 20 patients and there was no emergence of SI HIV-1

**TABLE 1**

Pattern of *in Vitro* Antibody Production in HIV-1-Infected Patients Divided by Clinical Stage

| Clinical stage | *n* | Anti-*env* Abs (% positive) | Anti-*core* Abs (% positive) |
|---|---|---|---|
| Recent seroconverter | 6 | 5 (83) | 0 (–) |
| Asymptomatic $\geqslant$500 $CD4^+/\mu$L | 15 | 15 (100) | 9 (60) |
| Asymptomatic $\geqslant$200 to $<$500 $CD4^+/\mu$L | 30 | 30 (100) | 9 (30) |
| Symptomatic (AIDS) | 8 | 8 (100) | 0 (–) |
| LTNPs | 20 | 12 (60) | 3 (15) |
| *P* value | | NS | 0.02 |

*Note.* NS, not significant.

**TABLE 2**

Characteristics of Long-Term Nonprogressors in Our Study Group

| Patients | IVAP | Viral isolation | Viral phenotype | CD4/$\mu$L | HIV-1 RNA |
|---|---|---|---|---|---|
| MI | gp160–p55–p24 | POS | NSI | 693 | 17 |
| DEL | gp160 | POS | SI | 625 | 41755 |
| RE | gp160 | POS | NSI | 1188 | 80 |
| FU | gp160 | POS | NSI | 610 | 4044 |
| RA | gp160 | POS | NSI | 700 | 50 |
| PE | gp160 | POS | NSI | 609 | 3087 |
| DAM | gp160 | POS | NSI | 611 | 568 |
| NA | gp160 | POS | NSI | 528 | 45531 |
| DAG | gp160 | NEG | NP | 729 | 1737 |
| AS | gp160 | NEG | NP | 558 | 3662 |
| GH | gp160 | NEG | NP | 900 | 91 |
| SA | gp160 | NEG | NP | 1116 | 2360 |
| FA | p24 | POS | NSI | 710 | 699 |
| BA | p55 | NEG | NP | 765 | 228 |
| ST | NEG | POS | NSI | 1041 | 147 |
| SE | NEG | POS | NSI | 787 | 2527 |
| VA | NEG | NEG | NP | 1305 | 825 |
| BU | NEG | NEG | NP | 795 | 132 |
| PA | NEG | NEG | NP | 744 | 500 |
| GR | NEG | NEG | NP | 1466 | 1532 |

Note. IVAP, *in vitro* antibody production; NEG, negative; POS, positive; NP, not possible; NSI, non-syncytium-inducing; SI, syncytium-inducing. HIV-1 RNA is expressed as number of copies/ml of plasma.

strains other than in 1 case who had a high level of viral copies in plasma (41,755/ml). With only 2 exceptions, the plasma viremia in LTNPs was below 5000 copies/ml (median 762; range 17–45,531). Median values (±standard error) of plasma viremia and CD4 cells in IVAP-positive and -negative subjects were 1218 ± 744 and 662 ± 230 copies/ml, and 696 ± 5 and 918 ± 174 cells/$\mu$l, respectively. It was not possible to find a statistical significance between these two groups in regards to viral load. We found a statistical correlation in $CD4^+$ cell counts between IVAP-positive and -negative patients ($P = 0.013$) and this correlation was also present in regard to anti-gp160 production ($CD4^+$ cells were 659 ± 64/$\mu$l in gp160-positive subjects and 791 ± 102 cells/$\mu$l in gp160-negative subjects; $P = 0.021$). Furthermore, there was no correlation between IVAP and the possibility of isolating HIV-1 from PBMC, even if the 2 anti-p24 producers were isolation-positive. Finally, the 5 healthy subjects who were HIV-1-seronegative showed a negative WB as well.

## DISCUSSION

The most relevant finding of our study is that LTNPs present a restricted pattern of IVAP in comparison to subjects in different stages of HIV-1 infection. LTNPs produced mainly anti-gp160 Abs. No other anti-*env* Abs were detected. On the contrary, anti-gp120 and anti-gp41 were demonstrated in 40 and 8% of the asymptomatic controls.

*In vitro* production of anti-*core* antibodies was absent in the majority of LTNPs, in seroconverters, in all of the subjects with laboratory or clinical evidence of disease progression, and also in a proportion of asymptomatic subjects with more than 500/$\mu$l $CD4^+$ cells, despite the presence of anti-p24 antibodies in the serum of all the patients enrolled in the study. A possible hypothesis in order to explain this observation is that anti-*core* antibody production may be restricted to B cells confined in lymphoid tissues, where HIV-1 is highly replicating, and in that those B cells fail to circulate.

LTNPs are reported to present a limited viral replication (5, 6) and our data confirmed this finding. In this case, the probability of detecting anti-HIV-1 Abs-secreting cells in peripheral blood may be reduced because of a low or rather absent antigenic stimulus. However, the low percentage of subjects showing anti-p24-secreting cells in the circulation does not support a relevant role of anti-*gag* Abs production in long-term nonprogression.

The presence of anti-gp160-secreting cells in the circulation of LTNPs was not significantly related to HIV-1 viral load, but the two subjects with high viremia (>40,000/ml) showed anti-gp160 production, and in one of these cases in association with Abs to p55 and p24. Moreover, anti-gp160 Abs production and IVAP *in toto* were related to a lower $CD4^+$ cell count in LTNPs. A possible explanation is that the production of anti-gp160 Abs in LTNPs is a signal of a less efficient control

of HIV-1 replication in patients with lower $CD4^+$ cell counts.

In conclusion, the presence of B cell clones secreting specific anti-HIV-1 Abs in the circulation does not seem to be related to long-term nonprogression of HIV-1 infection. The absence of anti-*gag* Abs-secreting B cell clones in peripheral blood of LTNPs might not only be a consequence of the loss of control of the viral replication as in advanced HIV-1 infection (11), but also of a low viral activity in resistant hosts with low antigenic stimulation. Longitudinal studies are needed to establish whether anti-gp160-secreting cells in the circulation represent a deficient control of viral replication more than a correlate of protection in LTNPs.

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